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Growth and Protein Phosphorylation in the Nb2 Lymphoma: Effect of Prolactin, cAMP, and Agents That Activate Adenylate Cyclase

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The Nb2 T lymphoma is unique in that these lymphocytes proliferate in response to prolactin as well as in response to interleukin-2. In this study, we have examined the responsiveness of the adenylate cyclase system in Nb2 cells and the role of this signaling system in regulating proliferation and protein phosphorylation. An analog of cAMP inhibited prolactin-stimulated proliferation and blocked a prolactin-induced decrease in protein phosphorylation. Forskolin, a potent activator of adenylate cyclase in T lymphocytes, did not elevate cAMP levels in Nb2 cells and was not an effective inhibitor of prolactin-induced proliferation. In fact, one preparation of forskolin stimulated proliferation of quiescent Nb2 cells. Like forskolin, prostaglandin E_2 did not stimulate cAMP production in Nb2 cells even though it increased cAMP in a preparation of rat peripheral blood lymphocytes. Cholera toxin appeared to ADP-ribosylate a stimulatory guanine nucleotide–binding protein in Nb2 cells, but the toxin did not increase intracellular levels of cAMP nor was it a potent anti-mitogenic agent. Pertussis toxin, an agent that can increase cAMP production through suppression of the inhibitory guanine nucleotide–binding protein, exerted only minor anti-proliferative actions on prolactin-stimulated Nb2 cells. These data suggest that cAMP inhibits Nb2 cell proliferation and prolactin-induced changes in protein phosphorylation but that the adenylate cyclase system in our clone of Nb2 cells responds poorly to agents that normally increase cAMP.

Key words: forskolin, cholera toxin, pertussis toxin, interleukin-2, T lymphocyte, G protein

The adenylate cyclase system generally mediates anti-proliferative signals in lymphocytes [1]. Indeed, multiple steps in the proliferation pathway may be negatively regulated by cAMP [2–4]. The adenylate cyclase system also may mediate anti-proliferative signals in Nb2 cells, a T lymphoma [5,6] that proliferates in response to prolactin (PRL) [7] and to interleukin-2 (IL-2) [6,8,9], because phosphodiesterase inhibitors [10,11] and cAMP analogs [10–12] consistently inhibit PRL-induced proliferation of these cells. An anti-proliferative role for adenylate cyclase is further supported by the fact that Nb2 cell proliferation is inhibited by cholera toxin [10–12], forskolin

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[10], and prostaglandin (PGE_2) [13], agents known to activate adenylate cyclase. However, recent studies [10,14] have questioned whether these latter agents act by increasing intracellular levels of cAMP. In one study [10], it was reported that concentrations of cholera toxin sufficient to inhibit proliferation do not increase cAMP. Moreover, a recent study [14] showed that high concentrations of forskolin do not elevate cAMP levels in Nb2 cells. Thus, whereas cAMP appears to exert anti-proliferative actions in Nb2 cells, there is some question whether the adenylate cyclase system plays a physiological role in these T lymphocytes.

In this report, we confirm the anti-proliferative actions in Nb2 cells of a cAMP analog and show that the analog can block a PRL-induced decrease in protein phosphorylation. Our results also show, however, that cAMP levels do not increase in response to forskolin, PGE_2, or cholera toxin. Forskolin and cholera toxin exert some weak anti-proliferative effects, but neither is a potent growth-inhibitory agent. In fact, one preparation of forskolin stimulates Nb2 cell proliferation. Pertussis toxin exerts only weak inhibitory actions. Taken together, these results suggest that cAMP can activate inhibitory pathways in Nb2 cells but that the inhibitory actions of forskolin, cholera toxin, and PGE_2 are not mediated through cAMP in our clone of cells.

MATERIALS AND METHODS

Materials

Ovine prolactin (PRL; NIH-P-I-2) was obtained from the Hormone Distribution Program, NIH (Bethesda, MD). Cholera toxin, cholera toxin B subunit, and pertussis toxin were purchased from List Biological Laboratories, Inc. (Campbell, CA). Forskolin was purchased from Calbiochem (La Jolla, CA) and from Sigma Chemical Co. (St. Louis, MO). PGE_2, 8-bromo-cAMP (8-Br-cAMP), Histopaque, Fischer's Medium for Leukemic Cells of Mice, and RPMI 1640 were purchased from Sigma. Fetal bovine serum was purchased from HyClone Laboratories, Inc. (Logan, UT) and horse serum was obtained from Flow Laboratories, Inc. (McLean, VA). [^{32}P]NAD and the cAMP radioimmunoassay kit were purchased from DuPont–New England Nuclear (Boston, MA). BCA protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL).

Cell Culture

The Nb2 cell clone (2A3) used in this study was grown in Fischer's medium supplemented with 10% fetal bovine serum, 10% horse serum (screened for low PRL content), 0.1 mM 2-mercaptoethanol, 100 U penicillin/ml and 100 μg streptomycin/ml and was maintained in a humidified atmosphere of CO_2/air (1:19) at 37°C as described by Tanaka et al. [15]. Approximately 24 h before addition of test substances, the Nb2 cells were growth arrested by removing fetal bovine serum from the medium (i.e., resuspended in "assay medium"). All experiments were performed in the presence of 10% horse serum except where noted. Cell number and cell diameter were determined using a Coulter model ZM counter and channelyzer (Coulter Electronics, Hialeah, FL). Cell analyses using the channelyzer show that Nb2 cells cultured in the "prolactin-free" horse serum decrease in size to a uniform diameter of approximately 9.5 μm. When stimulated with PRL, the cells move through the cell cycle in synchrony [13]. PEER cells, a human T-cell line [16], were kindly provided by Dr. A. Weiss (Howard Hughes Medical Institute, San Francisco, CA).

Protein Phosphorylation and Two-Dimensional Gel Electrophoresis

Phosphorylation studies were performed as described previously [9]. Briefly, Nb2 cells were growth arrested in assay medium 24 h before use in phosphorylation studies. The cells were then resuspended (5×10^5 cells/ml) in Fischer's medium in which 18 mM Hepes was substituted for the normal phosphate buffer. The medium was supplemented with 0.1% bovine serum albumin, complete amino acids for RPMI 1640 medium, 2 mM glutamine, 100 U of penicillin/ml, and 100 μg streptomycin/ml. One milliliter portions of the cell suspension were dispensed into a 24 well culture plate, and the cells were immediately pulsed with [^{32}P]Pi at 0.05–0.1 mCi/well. After incubation of the cells at 37°C in a humidified atmosphere of CO_2/air (1:19) for 0.5 h, test agents were added and the incubation was continued for 4 h. Phosphorylation was terminated by suspending the Nb2 cells in 10 ml of a wash buffer (4°C) containing 0.01 M sodium phosphate buffer (pH 7.4), 0.15 M NaCl, 4 mM EDTA, 10 mM NaF, 0.1 mM sodium vanadate, and 10 mM $Na_4P_2O_7$. The cells were collected by centrifugation and washed once more in the wash buffer. Washed pellets were subjected to freeze/thaw at least twice in 0.5 ml of lysis solution containing 850 μl lysis buffer, 100 μl sonication buffer, and 50 μl DNase as described by O'Farrell [17]. Isoelectric focusing was performed for 15–25 h at 400 V and for 1 h at 800 V in 130 × 3 mm 4% polyacrylamide gels containing 2% Biolytes (1.6% pH 5–7, 0.4% pH 3–10; Bio-Rad Laboratories, Richmond, CA). The second dimension was sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (4% stacking gel, 5–20% linear-gradient separating gel) as described by Laemmli [18]. The gels were dried and autoradiographed as described previously [9]. Pre-stained protein markers (Bio-Rad) were used for molecular weight (MW) determinations.

Isolation of Rat Peripheral Blood Lymphocytes (PBL)

Rats anesthetized with sodium pentobarbital were subjected to cardiac puncture and blood was collected into heparinized tubes. Lymphocytes isolated by density-gradient centrifugation with Histopaque were washed twice with RPMI 1640 supplemented with 10% fetal bovine serum and resuspended in the same medium. The cells were applied to a nylon-wool column according to the method of Julius et al. [19] and non-adherent cells were collected for determination of cAMP.

cAMP Determination

Nb2 (2A3) cells (3.6×10^7 cells/ml), rat PBL (3.6×10^7 cells/ml), and PEER cells (1.6×10^7 cells/ml) were suspended in Fischer's medium (pH 7.4) supplemented with 20 mM HEPES, 0.1 mM 3-isobutyl-1-methylxanthine, and 0.1% bovine serum albumin. Test reagents were added to 1 ml aliquots of the cell suspensions and the suspensions were incubated for 15 min at 37°C in a water bath. The reaction was terminated by heating the cell suspensions to 100°C over a flame and the samples were centrifuged at 500*g* to remove insoluble material. Supernatant fractions were assayed for cAMP using a cAMP radioimmunoassay kit according to the manufacturer's instructions. Samples were assayed in duplicate at two to three concentrations.

Analysis of ADP-Ribosylation

Membranes were isolated from approximately 1×10^8 Nb2 (2A3) cells by the method of Burns et al. [20]. The amount of membrane protein was determined using the BCA protein assay [21]. Activation of cholera toxin and pertussis toxin was accom-

plished by incubating the proteins in 25 mM potassium phosphate (pH 8.0), 25 mM dithiothreitol, and 1 mg bovine serum albumin/ml; incubation was at 37°C for 20 min [22,23]. Membranes (60 μg protein/ml) suspended in 100 mM potassium phosphate (pH 8.0), 12 mM thymidine, 1 mM $MgCl_2$, 0.5 mM EDTA, 5 mM HEPES, 1 mM ATP, 0.1 mM GTP, and 0.3 μM [^{32}P]NAD (800 Ci/mmol) were incubated with or without activated toxin (50 μg/ml) in a total reaction volume of 150 μl; incubation was at 30°C for 45 min [21]. In some incubations, 1 mM GTP was used to enhance labeling of stimulatory (G_s) and inhibitory (G_i) guanine nucleotide–binding proteins [24,25]. Membranes were collected by centrifugation at 15,000*g* for 6 min and washed once with 50 mM Tris-HCl (pH 7.5). Membrane proteins were solubilized by heating at 100°C for 5 min in 100 μl of 0.05 M Tris-HCl (pH 6.8), 2% (v/v) SDS, 2% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol. Radioactively labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (4% stacking, 10% separating gel) and autoradiography.

Statistical Analysis

The data were analyzed by one- or two-way ANOVA and pairwise comparisons between treatment means were performed by the Newman-Keuls test. All data are expressed as means ± S.E.M.

RESULTS

cAMP consistently has been shown to exert anti-proliferative actions in normal lymphocytes [1] and in Nb2 cells [10–12]. In accordance with these earlier findings, 8-Br-cAMP (1 mM) inhibited Nb2 cell proliferation in response to PRL (10 ng/ml; Fig. 1). It is generally believed that many of the actions of cAMP in eukaryotic cells are mediated through protein kinases; accordingly, Nb2 cell proteins phosphorylated in response to 8-Br-cAMP and PRL were analyzed by two-dimensional gel electrophoresis and autoradiography. Cells were exposed to test agents for 4 h, because previous studies [9,14] have demonstrated changes in protein phosphorylation after exposure of Nb2 cells

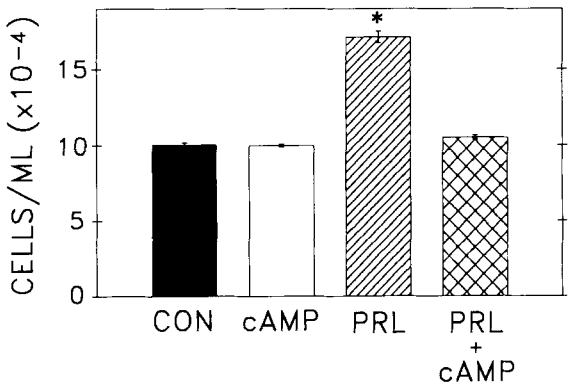

Fig. 1. Effect of 8-Br-cAMP on the proliferation of Nb2 cells. Growth-arrested cells were left untreated (CON) or exposed to 8-Br-cAMP (1 mM), PRL (10 ng/ml), and PRL + 8-Br-cAMP for 24 h. Cell number was quantified directly as described in Materials and Methods. The bars show the mean ± S.E.M. of three replicates in a representative experiment. *$P < 0.001$ when compared to the proliferation of untreated cells.

to PRL for 3–4 h. In the absence of 8-Br-cAMP, PRL decreased incorporation of radioactive phosphate into four proteins with pIs of approximately 5.7 and MWs ranging from 31,000 to 48,000 (Fig. 2). When PRL and 8-Br-cAMP were added together, PRL was unable to decrease phosphorylation of the three smaller proteins, but there was a decrease in the radioactivity associated with the largest protein (Fig. 2). Overall, the results confirm that 8-Br-cAMP is an anti-proliferative agent in Nb2 cells and extend these findings by showing that 8-Br-cAMP can inhibit early phosphorylation events induced by PRL.

In contrast to 8-Br-cAMP, forskolin, a potent activator of adenylate cyclase [26], exerted only weak anti-proliferative actions. At a high concentration (100 μM), forskolin (Sigma) inhibited PRL-induced proliferation by only 25% (Fig. 3). Moreover, this forskolin preparation did not affect proliferation of unstimulated Nb2 cells. The apparent unresponsiveness of Nb2 cells to forskolin could not be attributed to the preparation, because the same forskolin preparation potently inhibited proliferation of PEER cells (Fig. 3, inset), a human T-cell line. Interestingly, one preparation of forskolin (Lot 605861, Calbiochem) weakly inhibited PRL-induced Nb2 cell proliferation but stimulated the proliferation of quiescent cells (Fig. 4). A subsequent preparation (Lot 886164) from the same company did not exert any trophic effects (data not shown). Consistent with its limited anti-proliferative actions, forskolin did not stimulate cAMP production in Nb2 cells (Fig. 5). However, forskolin elevated cAMP in rat PBL and it induced a large increase in cAMP in PEER cells (Fig. 5). PGE_2, an agent that inhibits Nb2 cell proliferation [13], did not increase the levels of cAMP in Nb2 cells, but PGE_2 increased levels of cAMP in rat PBL and in PEER cells. Thus, although 8-Br-cAMP was an effective inhibitor of Nb2 cell proliferation, forskolin exerted only weak anti-proliferative actions, and neither forskolin nor PGE_2 induced a detectable increase in cAMP.

Cholera toxin, a substance that activates adenylate cyclase through ADP-ribosylation of G_s, inhibited Nb2 cell proliferation (Fig. 6) but only at concentrations higher than those reported in previous studies [10–12]. Moreover, the cholera toxin B subunit also was an effective inhibitor despite the fact that the B subunit does not stimulate ADP-ribosylation. It is possible, therefore, that the anti-proliferative effects of cholera toxin in these cells were not mediated through cAMP but were related to binding of the B subunit. Previous studies have shown that the B subunit can inhibit T-cell proliferation [27] and promote B-cell isotype differentiation [28]. In this regard, cholera toxin (100 ng/ml) did not induce any detectable increase in cAMP (data not shown) in these cells. The cholera toxin preparation was active, because it stimulated incorporation of radioactivity into proteins with MWs of 45,000 and 41,000 (Fig. 7) when Nb2 cell membranes were incubated with [^{32}P]NAD. The MWs of these proteins are consistent with those of G_s and G_i, respectively; thus, the data support those of previous studies [10,11] showing that cholera toxin ADP-ribosylates G_s and G_i in Nb2 cells. No ADP-ribosylation was obtained using the B subunit of cholera toxin (data not shown).

PRL-stimulated proliferation was only slightly inhibited by pertussis toxin (0.1–100 ng/ml; Fig. 8), a substance that ADP-ribosylates and inactivates G_i [29]. The inhibition did not appear to be specific for PRL-stimulated proliferation, because at concentrations effective in inhibiting PRL-stimulated proliferation, pertussis toxin also affected quiescent cells (Fig. 8). These findings are consistent with results reported by Larsen and Dufau [12] but do not support the greater inhibition observed by Too et al. [11]. Although it was not a potent anti-proliferative agent in the Nb2 cells, pertussis toxin appeared to ADP-ribosylate a protein with a MW similar to that of G_i (Fig. 7).

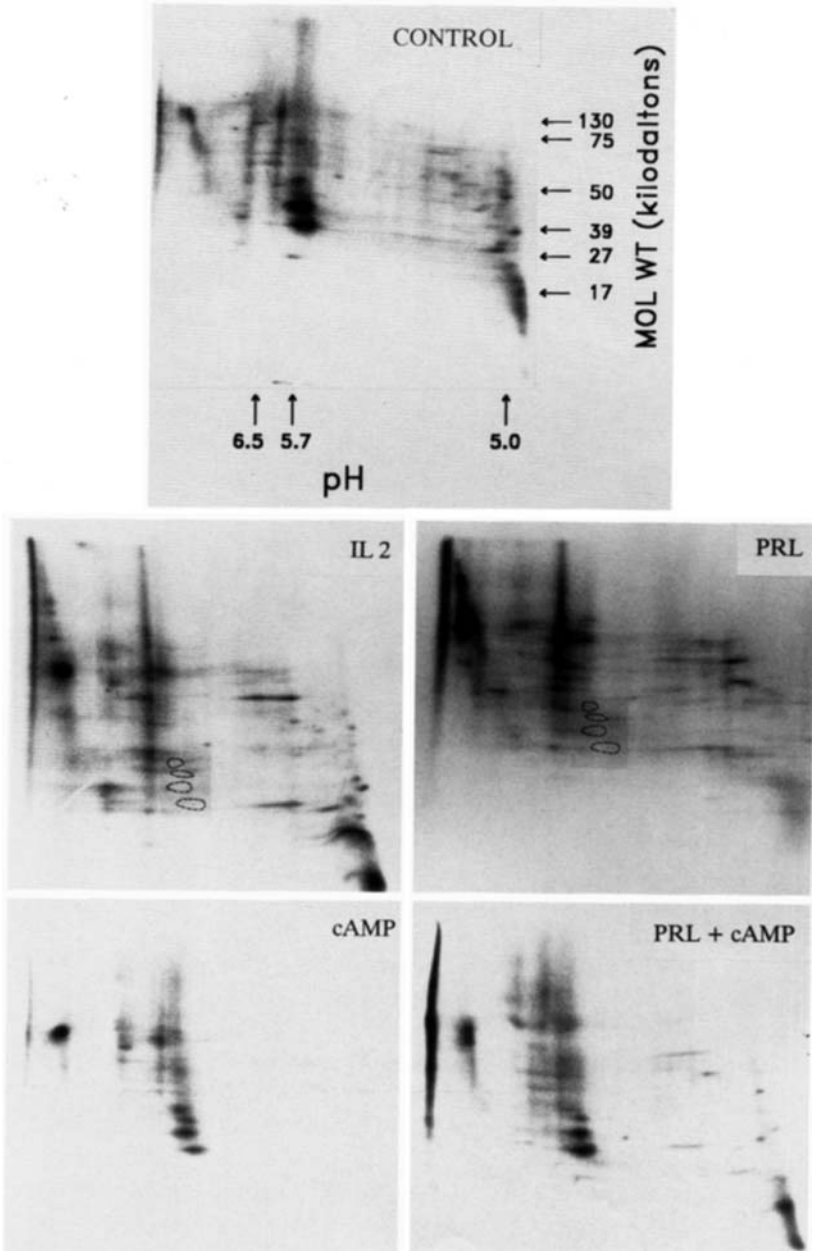

Fig. 2. Autoradiographs of [^{32}P]labeled proteins analyzed by two-dimensional electrophoresis. Growth-arrested Nb2 cells were left untreated (control) or were exposed to IL-2 (10 U/ml), PRL (10 ng/ml), 8-Br-cAMP (1 mM), or PRL + 8-Br-cAMP for 4 h in the presence of [^{32}P]Pi. The areas outlined on the IL-2 and PRL autoradiographs show the positions of four radioactive proteins visible on the autoradiograph of proteins from untreated cells (control). The acidic range (pI 5.0) of each gel is located at **right** and the basic end (pI 7.0) is located at the **left.** The separating gel for SDS-polyacrylamide electrophoresis was a linear gradient of 5–20% acrylamide. MWs ($\times 10^{-3}$) of standards are shown at the **right** and pI markers are shown at the **bottom** of the Control gel. The results shown are representative of three separate experiments.

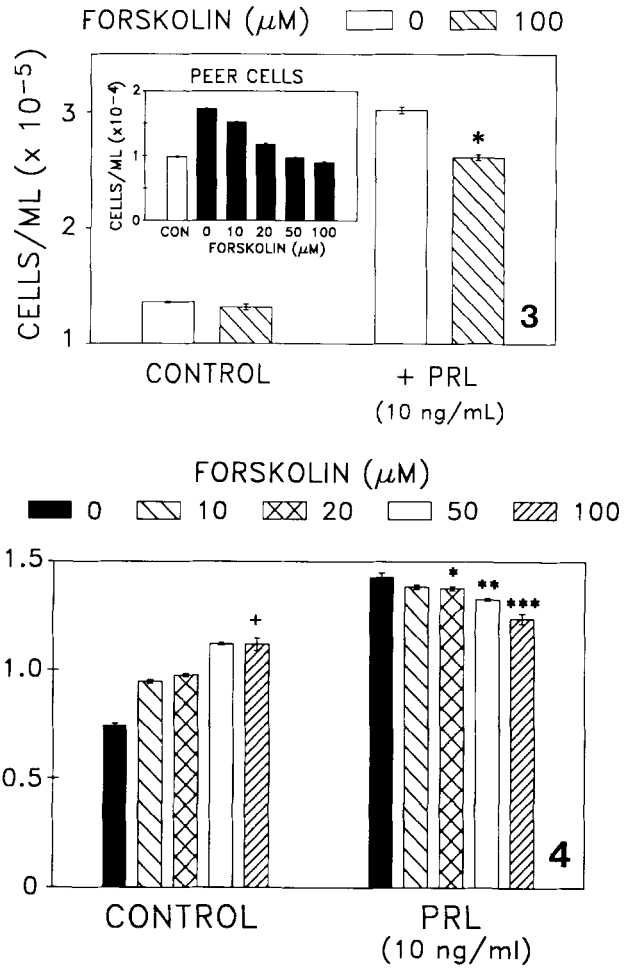

Fig. 3. Effect of forskolin (Sigma) on the proliferation of Nb2 cells and PEER cells (**inset**). Nb2 cells were exposed to forskolin (100 μM) immediately before the addition of zero PRL (control) or 10 ng PRL/ml for 24 h. PEER cells were distributed into 1 ml wells at a concentration of 9.8×10^4 cells/ml (CON) and were grown in the absence (0) or presence (10–100 μM) of forskolin for 24 h. The bars show the mean ± S.E.M. of six replicates in a representative experiment. *P <0.01 when compared with the proliferation of cells exposed only to PRL.

Fig. 4. Proliferation of Nb2 cells in response to a preparation of forskolin (Lot 605861) obtained from Calbiochem. The cells were exposed to forskolin (10–100 μM) immediately before addition of zero PRL (control) or 10 ng PRL/ml for 24 h. The bars show the mean ± S.E.M. of three replicates in a representative experiment. *P <0.05; **P <0.01; ***P <0.001 when compared with the proliferation of cells exposed only to PRL. +, P <0.001 for all concentrations of forskolin when compared with untreated cells.

DISCUSSION

Recent studies [10–12] have shown that cAMP is an anti-proliferative agent in Nb2 cells. In the present studies, we confirmed the anti-proliferative actions of a cAMP analog and have further shown that the analog can block a PRL-induced decrease in phosphorylation, an action that occurs within 4 h. IL-2 decreases phosphorylation of the

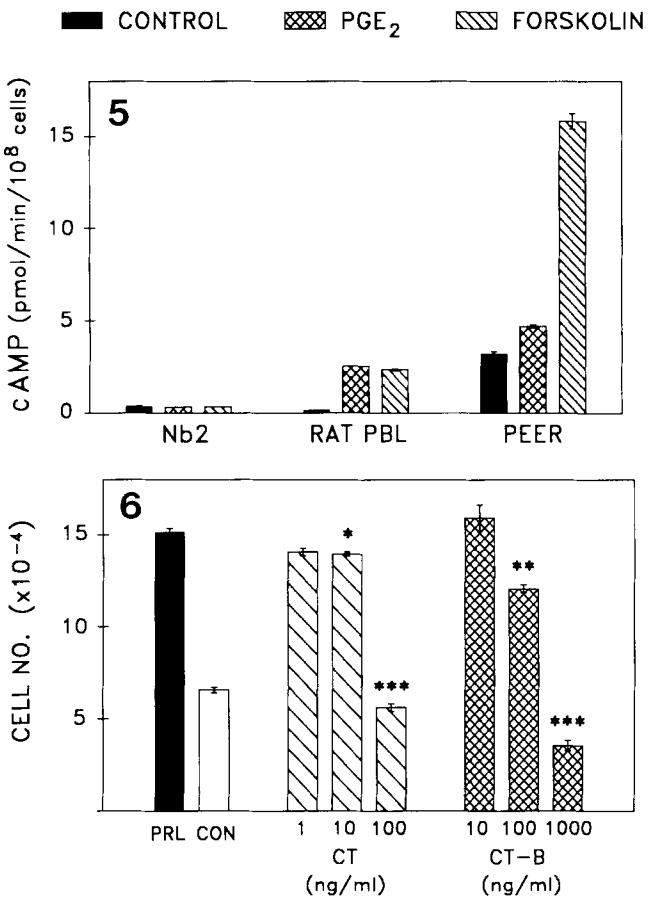

Fig. 5. Effect of forskolin and PGE_2 on the production of cAMP in Nb2 cells (Nb2), rat peripheral blood lymphocytes (rat PBL), and PEER cells. Untreated cells (control) and cells exposed to PGE_2 (20 μM) and forskolin (100 μM) were incubated for 15 min at 37°C, and cAMP was determined by radioimmunoassay. The bars show the mean ± S.E.M. of three replicates in a representative experiment.

Fig. 6. Effect of cholera toxin (CT) and the B subunit of cholera toxin (CT-B) on the proliferation of Nb2 cells. The cells were exposed to cholera toxin or to its B subunit immediately before addition of PRL (10 ng/ml) for 24 h. Groups composed of untreated cells (CON) and cells exposed only to PRL (PRL) showed cell numbers in nonproliferating and maximally proliferating groups, respectively. The bars show the mean ± S.E.M. of three replicates in a representative experiment. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ when compared to the proliferation of cells exposed only to PRL.

same proteins that are responsive to PRL; thus these proteins are components of pathways regulated by two separate mitogens. These data support the report by Too et al. [11] showing that phosphodiesterase inhibitors and cAMP inhibit the actions of PRL and IL-2 in Nb2 cells.

It is less clear, however, whether activation of the adenylate cyclase system in Nb2 cells produces inhibitory quantities of cAMP. Forskolin, a diterpene that directly activates adenylate cyclase [26], did not elevate cAMP in this study or in two previous studies [10,14]. The apparent unresponsiveness to forskolin in our study was unexpected, because the preparation increased cAMP in rat PBL and in PEER cells, and forskolin has been shown to elevate cAMP in rat thymocytes [30]. The modest anti-proliferative

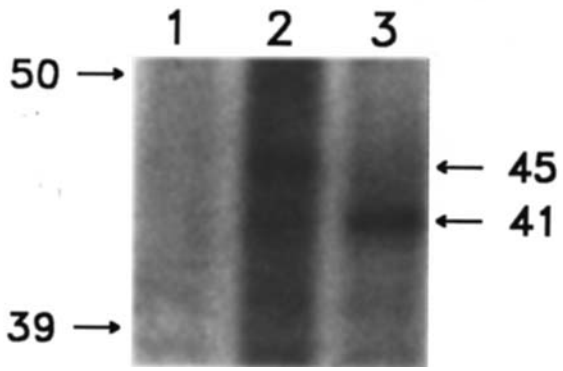

Fig. 7. G proteins in Nb2 cells ADP-ribosylated in response to cholera toxin and to pertussis toxin. Membrane protein (9 μg) was incubated with [^{32}P]NAD in the absence of toxin (**lane 1**) and in the presence of cholera toxin (50 μg/ml, **lane 2**) and pertussis toxin (50 μg/ml, **lane 3**). Radioactive proteins were analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and autoradiography. The positions of MW markers (MW $\times 10^{-3}$) are shown at **left**, and the MWs ($\times 10^{-3}$) of proteins radioactively labeled in response to cholera toxin or pertussis toxin are shown at **right**.

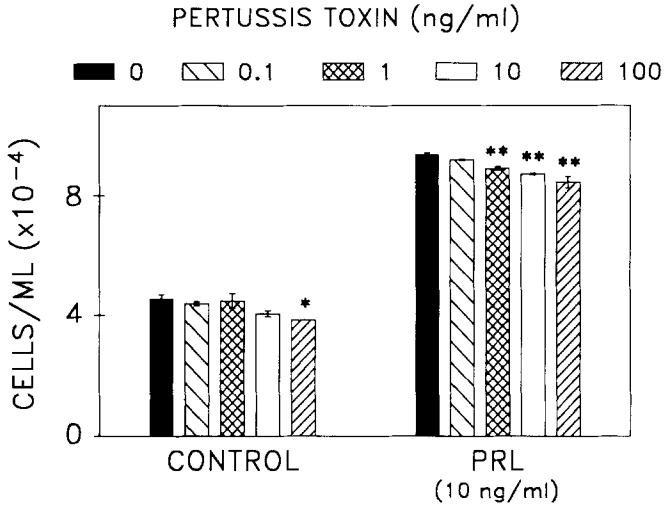

Fig. 8. Effect of pertussis toxin on the proliferation of Nb2 cells. The cells were exposed to pertussis toxin immediately before the addition of zero PRL (control) or 10 ng PRL/ml for 24 h. The bars show the mean ± S.E.M. of three replicates in a representative experiment. *P <0.025; **P <0.01 when compared to proliferation in groups not exposed to pertussis toxin.

effects of forskolin observed in this study and in a previous study [10] may reflect small increases in the intracellular levels of cAMP; however, it has been shown [31] that, independent of cAMP, forskolin can inhibit T-cell mitogenesis by decreasing voltage-gated K^+ conductance. A similar mechanism may be operative in Nb2 cells, because inhibitors of voltage-gated K^+ conductance decrease PRL-induced actions in these cells [32]. Irrespective of forskolin's mechanism of action, it appears that the adenylate cyclase system in Nb2 cells, or at least in our clone, is less able to respond to forskolin. Decreased production of cAMP in response to forskolin may account for the ability of

one forskolin preparation to stimulate proliferation of quiescent Nb2 cells. A lymphotrophic contaminant in this preparation probably would not be detected if high intracellular levels of cAMP were present to block proliferation, as in PEER cells. The possible presence of a lymphotrophic agent in a forskolin preparation is of some interest, because it seems unlikely that the putative agent is related to known peptide lymphokines. Overall, the data suggest that our Nb2 cell clone is relatively unresponsive to forskolin and that a substance that co-purifies with forskolin may exert growth-promoting actions.

An adenylate cyclase which is less responsive to some stimulatory agents would account for the results obtained using cholera toxin as well as those obtained in forskolin experiments. In particular, low responsivity would explain the weak anti-proliferative actions of cholera toxin and the inability of the toxin to increase levels of cAMP. However, a less-responsive adenylate cyclase, if it exists in our cell clone, may not be characteristic of all Nb2 cell subsets, because Pines et al. [10] have shown that high concentrations of cholera toxin increase cAMP. The anti-proliferative actions observed using cholera toxin may have been due in part to binding of the B subunit, because this subunit decreased PRL-induced proliferation. Moreover, the B subunit has been shown to inhibit T-cell proliferation [27]. The lower anti-proliferative activity of the B subunit relative to the intact toxin may suggest that cAMP also plays a role or that the A subunit directly enhances the actions of the B subunit. In any case, as with forskolin and PGE_2, it is unclear whether cAMP mediates the actions of cholera toxin in Nb2 cells.

In the present study, pertussis toxin inhibited PRL-induced proliferation only to a small extent, and the toxin affected quiescent cells as well as those exposed to PRL. The preparation of pertussis toxin appeared to ADP-ribosylate a G protein(s), but it is unknown whether the limited inhibition observed in this study involved G proteins, because, as with cholera toxin, the binding subunit of pertussis toxin exerts independent actions in lymphocytes [33]. Overall, the data do not suggest that pertussis toxin–sensitive G proteins play an important role in mediating the growth-promoting actions of PRL.

Generalizations regarding signalling pathways in Nb2 cells are difficult to make due to the apparent differences in responsiveness of the subsets of Nb2 cells used in various laboratories. Inhibitors of phosphodiesterase increase cAMP in Nb2 cells [14] and cAMP exerts anti-proliferative actions [10–12], but the adenylate cyclase system in some subsets of Nb2 cells appears to be relatively unresponsive to a number of stimulatory agents [10,14]. Moreover, changes in intracellular cAMP do not always reflect the inhibitory activity of these agents [10]. This appears to be particularly true of the Nb2 cell clone used in this study. Pertussis toxin–sensitive G proteins do not appear to be good candidates as direct mediators of the actions of PRL, because consistent inhibitory effects have not been reported with pertussis toxin. Changes in phosphorylation have been reported in this and previous studies [9,14], but these changes occurred after a period of hours, and thus are not the initial events following activation of the PRL receptor. Therefore, additional studies are needed to identify the initial intracellular events mediating the actions of PRL.

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